Significance of Peptide Transporter 1 in the Intestinal Permeability of Valacyclovir in Wild-Type and *PepT1* Knockout Mice

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ABSTRACT

The purpose of this study was to quantitatively determine the contribution of PepT1 [peptide transporter 1 (SLC15A1)] to the intestinal permeability of valacyclovir, an ester prodrug of the antiviral drug acyclovir. In situ single-pass intestinal perfusions were employed (pH 6.5 × 90 minutes) to assess the effective permeability (P_{eff}) of 100 μM [^{3}H]valacyclovir in wild-type and *PepT1* knockout mice. Acyclovir pharmacokinetics was also evaluated after oral administration of 25 nmol/g valacyclovir. In wild-type mice, jejunal uptake of valacyclovir was best described by both saturable (K_m = 10.2 mM) and nonsaturable components where the saturable pathway accounted for 82% of total transport. Valacyclovir P_{eff} was 2.4 × 10^{-4} cm/s in duodenum, 1.7 × 10^{-4} cm/s in jejunum, 2.1 × 10^{-4} cm/s in ileum, and 0.27 × 10^{-4} cm/s in colon. In *Pept1* knockout mice, P_{eff} values were about 10% of that in wild-type animals for these small intestinal segments. Valacyclovir P_{eff} was similar in the colon of both genotypes. There were no differences in valacyclovir P_{eff} between any of the intestinal segments of *PepT1* knockout mice. Valacyclovir P_{eff} was significantly reduced by the dipeptide glycylsarcosine and the aminocephalosporin cefadroxil, but not by the amino acids L-valine or L-histidine, the organic acid *p*-aminohippurate, or the organic base tetraethylammonium (all at 25 mM). PepT1 ablation resulted in 3- to 5-fold reductions in the in vivo rate and extent of valacyclovir absorption. Our findings conclusively demonstrate, using in situ and in vivo validations in genetically modified mice, that PepT1 has a major influence in improving the oral absorption of valacyclovir.

Introduction

Limited options are currently available to breach the permeability barriers of hydrophilic drugs. Bioconvertable derivatives with increased lipophilicity have shown some success in improving the intestinal permeability and uptake of hydrophilic compounds (Jana et al., 2010). Alternatively, a novel type of prodrug, designed to target intestinal transporters, is emerging as an attractive approach for permeability enhancement (Li et al., 2008; Varma et al., 2010). Epithelial cells in the small intestine express a variety of influx transporters including peptide transporters, amino acid transporters, organic anion transporting polypeptides, and others, which function to actively uptake their endogenous substrates (Brandsch et al., 2008; König, 2011; Thwaites and Anderson, 2011). Targeted prodrugs of poorly permeable compounds are designed to mimic the key structural features of endogenous substrates and, as a result, take advantage of uptake carriers present in the intestine to improve the absorption of parent drug.

Among the oral delivery candidates available for targeted prodrugs, peptide transporter 1 (PepT1) is particularly promising due to some favorable properties. PepT1 (SLC15A1) is a proton-coupled oligopeptide transporter abundantly expressed in the apical membranes of small intestinal epithelial cells of both human and mouse (Groneberg et al., 2001; Jappar et al., 2010). The physiologic role of PepT1 is to facilitate the uptake of dietary dipeptides/tripeptides after protein digestion in the intestine (Daniel, 2004). As a high-capacity low-affinity influx transporter, PepT1 couples the active uptake of its substrate against the concentration gradient with the downhill uptake of proton into enterocytes (Brandsch et al., 2008; Rubio-Aliaga and Daniel, 2008). The apical location, abundant intestinal expression, and high-capacity low-affinity nature of uptake suggest that PepT1 can transport targeted prodrugs efficiently without being easily saturated even at relatively high oral doses. Moreover, PepT1 transports structurally diverse compounds including dipeptides/tripeptides such as glycylsarcosine and glycyl-L-proline, β-lactam antibiotics such as cefadroxil and cephalexin, angiotensin converting enzyme inhibitors such as captopril and enalapril, the anticancer drug bestatin, and the photosensitizing agent 5-aminolevulinic acid (Smith et al., 2013). This broad substrate specificity indicates that intestinal PepT1 may be a versatile delivery target for prodrugs with distinct structures.

Various PepT1 targeted prodrugs, which are commonly synthesized by linking poorly permeable active drugs with some amino acid or dipeptide carriers, are under intensive investigation (Tsume et al., 2008; Sun et al., 2010; Gupta et al., 2011; Yan et al., 2011). Among these prodrugs, valacyclovir is viewed as the prototype compound with a large available database of mechanistic, absorption, and disposition properties. Valacyclovir, an L-valyl ester prodrug of the potent antiviral agent acyclovir, is orally administered for the treatment and prophylaxis of herpes simplex, varicella zoster, and cytomegalovirus infection. In human subjects, orally administered valacyclovir has a systemic availability of 54% for acyclovir, a value 3- to 5-times higher than that of orally administered

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ABBREVIATIONS: ATB$^{0,+}$, amino acid transporter (SLC6A14); GlySar, glycylsarcosine; HPLC, high-performance liquid chromatography; OAT, organic anion transporter; OCT, organic cation transporter; PAH, *p*-aminohippurate; P_{eff}, effective permeability; PepT1, peptide transporter 1 (SLC15A1); PhT1, peptide histidine transporter (SLC15A4); PhT2, peptide histidine transporter 2 (SLC15A3); QC, quality control.

acyclovir (Soul-Lawton et al., 1995). Although the uptake of valacyclovir was clearly shown to increase in different PepT1-expressing cells (de Vrueh et al., 1998; Ganapathy et al., 1998; Han et al., 1998; Guo et al., 1999; Balimane and Sinko, 2000; Bhardwaj et al., 2005), this prodrug was also found to be a substrate for the peptide transporter 2 (SLC15A2) (Ganapathy et al., 1998), the amino acid transporter ATB[0,+] (SLC6A14) (Hatanaka et al., 2004), the peptide histidine transporter 1 PhT1 (SLC15A4) (Bhardwaj et al., 2006), and potentially some organic anion transporters (OATs) and organic cation transporters (OCTs) (Sinko and Balimane, 1998; Takeda et al., 2002). Since some of these transporters coexist in the intestine, it is crucial to quantify the contribution of PepT1 in mediating the intestinal absorption of valacyclovir. Unfortunately, studies demonstrating the quantitative importance of PepT1 in improving the intestinal absorption of valacyclovir are completely lacking.

With this in mind, the primary aim of this study was to evaluate the quantitative significance of PepT1 on the permeability of valacyclovir, using in situ single-pass perfusions, in different intestinal regions of wild-type and *PepT1* knockout mice, and to characterize the in vivo oral absorption of valacyclovir, and systemic exposure of acyclovir, in both genotypes after oral dosing of prodrug. By combining these in situ and in vivo approaches in genetically modified mice, we clearly demonstrate the rationale for designing PepT1 targeted prodrugs for enhanced oral drug absorption in the gastrointestinal tract.

Materials and Methods

Animals. Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Gender-matched wild-type and *PepT1* knockout mice, 8–10 weeks of age, were used for all experiments (Hu et al., 2008). The mice were kept in a temperature-controlled environment with 12-hour light/dark cycles, and received a standard diet and water ad libitum (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI).

Materials. Valacyclovir hydrochloride, acyclovir, glycylsarcosine (GlySar), cefadroxil, TEA, *p*-aminohippurate (PAH), L-histidine, L-valine, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

In Situ Single-Pass Jejunal Perfusions. Prior to experimentation, wild-type and *PepT1* knockout mice were fasted overnight (approximately 16 hours) with free access to water. As reported previously (Adachi et al., 2003; Jappar et al., 2010), the animals were anesthetized with sodium pentobarbital (40–60 mg/kg i.p.) and placed on a warming pad to maintain body temperature. Isopropyl alcohol was used to sterilize the abdominal area and a 1.5-cm midline incision was made longitudinally to expose the small intestine. An 8-cm segment of proximal jejunum was isolated (i.e., approximately 2 cm distal to the ligament of Treitz), and incisions were then made at the proximal and distal ends. Glass cannulas (2.0 mm outer diameter), attached to flexible polyvinyl chloride tubing, were inserted into both ends of the jejunum and secured with silk sutures. The inlet tubing was connected to a 20-ml syringe placed on a perfusion pump (Model 22; Harvard Apparatus, South Natick, MA) and the outlet tubing was placed in a collection vial. The perfusion buffer (pH 6.5), containing 10 mM MES, 135 mM NaCl, 5 mM KCl, and 100 μM valacyclovir with an osmolarity of 290 mOsm/l, was incubated at 37°C to maintain body temperature and then pumped through the jejunal segment at 0.1 ml/min for 90 minutes. After a 30-minute perfusion period to ensure steady-state conditions (based on previous experience), the exiting perfusate was then collected at 10-minute intervals for 1 hour (i.e., at 40, 50, 60, 70, 80, and 90 minutes). Upon completion, the length of intestinal segment perfused was accurately measured. A perfusate pH of 6.5 was chosen for these studies because it represents the pH of maximal uptake for valacyclovir and because it reflects the physiologic pH of the intestine in rodent and human.

For concentration-dependent studies, perfusate concentrations of valacyclovir varied over a wide range (0.01–50 mM) to assess the saturable transport kinetics of prodrug in wild-type mouse jejunum. For specificity studies, the jejunum of wild-type mice was coperfused with 100 μM valacyclovir and 25 mM of potential inhibitors such as GlySar, cefadroxil, TEA, PAH, L-histidine,

or L-valine. Studies were also performed in which active drug, 100 μM acyclovir, was perfused in the jejunum of wild-type and *PepT1* knockout mice.

In Situ Single-Pass Segment-Dependent Perfusions. To characterize the effective permeability of valacyclovir in different intestinal regions, studies were performed in which the duodenum, jejunum, ileum, and colon were perfused simultaneously in wild-type and *PepT1* knockout mice. Thus, in these studies, a 2-cm segment of duodenum (i.e., approximately 0.25 cm distal to the pyloric sphincter), an 8-cm segment of jejunum (i.e., approximately 2 cm distal to the ligament of Treitz), a 6-cm segment of ileum (i.e., approximately 1 cm proximal to the cecum), and a 3-cm segment of colon (i.e., approximately 0.5 cm distal to the cecum) were perfused as previously described (Dahan et al., 2009; Jappar et al., 2010).

Bioconversion of Valacyclovir. The extent of valacyclovir conversion to acyclovir, after passing through the enterocyte, was evaluated experimentally by obtaining portal vein blood just before the end of a 90-minute perfusion. The blood samples were then immediately transferred to a 7.5% EDTA–containing centrifuge vial and centrifuged at 3000g for 3 minutes at room temperature. A 100-μl aliquot of plasma was collected and deproteinated by mixing the sample with 200 μl of acetonitrile. The mixture was vigorously vortex-mixed for 1 minute and then centrifuged at 15,000g for 10 minutes. The clear supernatant was transferred to a clean centrifuge vial, blown dry under vacuum, and reconstituted in 100 μl of mobile phase, 50 μl of which was injected directly into the high-performance liquid chromatography (HPLC) system.

Analytical Methods. Perfusate and plasma samples were assayed for valacyclovir and acyclovir using an HPLC system consisting of a Waters 616 pump, 717 autosampler, and 2475 multi λ fluorescent detector (excitation $\lambda =$ 270 nm, emission $\lambda =$ 360 nm) (Waters Inc., Milford, MA). A Waters ODS-3 column (250 $\times$ 4.6 mm, 5 μM), fitted with a refillable guard column, was used for chromatographic separation. The mobile phase consisted of the following: (A) a 5% organic phase, with 0.1% v/v trifluoroacetic acid in acetonitrile; and (B) a 95% aqueous phase, with 0.1% v/v trifluoroacetic acid in water pumped isocratically at 1 ml/min ambient temperature. All perfusate samples were centrifuged at 15,000g for 10 minutes and 50-μl aliquots of clear supernatant were injected directly into the HPLC system. Under these conditions, the retention times of acyclovir and valacyclovir were 4.6 minutes and 13.4 minutes, respectively.

Because of assay interference by PAH, the mobile phase was changed for those studies in which this compound was coperfused with valacyclovir in the jejunum. Specifically, a gradient elution was applied in which the mobile phase was increased as a step function from 1% A and 99% B (0–13 minutes) to 45% A and 55% B (13–20 minutes), and then back to 1% A and 99% B (20–35 minutes). Under these conditions, the retention times of acyclovir and valacyclovir were 11.5 minutes and 20.8 minutes, respectively, and 8.8 minutes for PAH.

HPLC Method Validation. The HPLC method was thoroughly validated in terms of specificity, linearity and sensitivity, precision and accuracy, and stability. With respect to specificity, endogenous compound interference was evaluated by analyzing blank perfusate collected from at least six different batches. Linearity was evaluated by preparing calibration standards of 2, 5, 10, 20, 50, 100, and 200 μM for valacyclovir and 0.5, 1, 2.5, 5, 10, 20, and 50 μM for acyclovir. The lower limit of quantification was calculated as the lowest concentration giving a peak signal to noise ratio $\geq$10. To assess the assay precision and accuracy, quality control (QC) samples of high, medium, and low concentrations were prepared for valacyclovir (200, 50, and 5 μM, respectively) and acyclovir (50, 10, and 1 μM, respectively). Intraday precision and accuracy were evaluated by analyzing the QC samples in triplicate, and interday precision and accuracy were evaluated by analyzing the QC samples in triplicate on 3 consecutive days. Assay precision was calculated as standard deviation divided by the mean, and accuracy as observed concentration divided by the spiked concentration. Stability of the QC samples was evaluated after storage for one month at 4°C and after 4 hours at room temperature, and was calculated as (observed concentration − spiked concentration) divided by the spiked concentration. Values for precision and accuracy, as well as stability, were expressed as percentages.

Oral Absorption of Valacyclovir. Wild-type and *PepT1* knockout mice were fasted overnight for approximately 16 hours before each experiment. After dissolving appropriate amounts of ^{3}H-labeled and unlabeled valacyclovir in normal saline, an oral dose of 25 nmol/g valacyclovir was administered by

gastric gavage (200 μl, 10 μCi per mouse). Serial blood samples (approximately 20 μl) were then collected via tail nicks at 0, 5, 15, 30, 45, 60, 90, 120, and 180 minutes after dosing. Mice were returned to their cages between blood sampling and had free access to water. The blood samples were transferred to 0.2-ml microcentrifuge tubes containing 7.5% potassium EDTA and centrifuged at 3000 g for 3 minutes, ambient temperature. A 5- to 10-μl aliquot of plasma was then transferred to a scintillation vial and 6 ml CytoScint scintillation fluid (MP Biomedicals, Solon, OH) was added to the sample. Radioactivity of the plasma sample was measured on a dual-channel liquid scintillation counter (Beckman LS 6000 SC; Beckman Coulter Inc., Fullerton, CA).

Data Analysis. The effective permeability (P_{eff}) of valacyclovir was estimated according to the complete radial mixing (parallel tube) model in Eq. 1 (Komiya et al., 1980; Kou et al., 1991):

$$P_{eff} = \frac{-Q_{in} \times \ln(C_{out}'/C_{in})}{2\pi RL} \quad (1)$$

where Q_{in} is the fixed inlet flow rate of 0.1 ml/min, C_{out}' is the water flux-corrected outlet concentration of valacyclovir as defined in Eq. 2, C_{in} is the inlet concentration of valacyclovir, R is the intestinal radius (0.1 cm), and L is the length of the perfused intestinal segment, respectively.

The water flux-corrected outlet concentration of valacyclovir C_{out}' was calculated as:

$$C_{out}' = (C_{out,VACV} + C_{out,ACV}) \times (Q_{out}/Q_{in}) \quad (2)$$

where $C_{out,VACV}$ and $C_{out,ACV}$ are the uncorrected concentrations of valacyclovir and acyclovir, respectively, in outlet perfusate, and Q_{out} and Q_{in} are the perfusate flow rates entering and leaving the intestinal segment, respectively, as measured gravimetrically (Sutton et al., 2001). The C_{out}' correction included both valacyclovir and acyclovir because of the luminal hydrolysis of valacyclovir, which was also reported during intestinal perfusion studies in rat (Sinko and Balimane, 1998). Moreover, luminally formed acyclovir did not affect the estimation of valacyclovir P_{eff} because its jejunal permeability was negligible compared with that of prodrug in wild-type mice (see *Results*).

The concentration-dependent uptake (V) of valacyclovir in jejunum of wild-type mice was best fitted by an equation consisting of a Michaelis-Menten term and a nonsaturable linear term (Eq. 3):

$$V = P_{eff} \times C_{in} = \frac{V_{max} \times C_{in}}{K_m + C_{in}} + K_d \times C_{in} \quad (3)$$

where V_{max} is the maximal rate of carrier-mediated uptake, K_m is the Michaelis constant referenced to inlet concentrations of valacyclovir, and K_d is the first-order rate constant for nonsaturable uptake.

After oral administration of valacyclovir, the pharmacokinetics of acyclovir was analyzed using a noncompartmental approach (WinNonlin, version 5.3; Pharsight Inc., Mountainview, CA). Specifically, the area under the acyclovir plasma concentration-time curve was calculated, from time 0 to 180 minutes, by linear trapezoidal rule. In addition, the peak plasma concentration (C_{max}) and time to reach this peak concentration (T_{max}) were obtained from observed values.

The data were reported as mean ± S.E. unless otherwise stated. Unpaired two-sample t tests were used to compare statistical differences between wild-type and *PepT1* knockout mice. Statistical differences between three or more groups were evaluated by one-way analysis of variance followed by Dunnett's test for pairwise comparisons with the control group or by Bonferroni's test for pairwise comparisons between multiple groups (Prism version 4.0; GraphPad Software Inc., La Jolla, CA). A P value ≤ 0.05 was considered statistically significant. Nonlinear regression analyses were performed using Prism version 4.0 software, in which goodness of fit was determined by the coefficient of determination (r^2), by the S.E. of estimated parameters, and by visual inspection of the residuals.

Results

HPLC Method Validation. Figure 1A shows a representative chromatogram of jejunal blank perfusate (i.e., without drug being present during the 90-minute perfusion) and clearly demonstrates that no endogenous substances were present that might interfere with the analysis of acyclovir or valacyclovir. Calibration curves of valacyclovir and acyclovir were constructed by least-squares linear regression for the peak areas versus spiked concentrations of each compound, and correlation coefficients were ≥0.999 for all analyses. The lower limit of quantification was 0.2 and 0.1 μM for valacyclovir and acyclovir, respectively. The precision and accuracy of the assay method were excellent. We found that the intraday and interday precision averaged ≤9% and the accuracy ranged ±6% for both compounds. With respect to chemical stability, the QC samples varied by less than 5% for both valacyclovir and acyclovir under the conditions studies.

Bioconversion of Valacyclovir. Figure 1, B–E, demonstrates that valacyclovir had substantial degradation in the jejunal and ileal segments, and some degradation in the duodenal and colonic segments, during intestinal perfusion. As a result, both acyclovir and valacyclovir needed to be measured in the outlet perfusate samples (i.e., all drug-related species) so that an accurate estimation of valacyclovir P_{eff} was possible. Moreover, as shown in Fig. 1F, the chromatographic peak corresponding to valacyclovir was completely absent from the portal vein plasma sample, indicating that the prodrug was rapidly and completely degraded to acyclovir during its passage through the enterocytes.

In Situ Single-Pass Jejunal Perfusion Studies. The jejunal uptake of valacyclovir was studied in wild-type mice as a function of perfusate concentration to evaluate the potential for capacity-limited transport kinetics. As observed in Fig. 2, the uptake of valacyclovir was composed of both saturable and linear components. The transport parameters were estimated as V_{max} = 1.4 ± 0.5 nmol/cm^2 per second, K_m = 10.2 ± 4.6 mM, and K_d = 3.0 ± 0.6 × 10^{-5} cm/s (r^2 = 0.996), in which the saturable component was responsible for 82% of the total uptake of valacyclovir [i.e., calculated as: (V_{max}/K_m) / ($V_{max}/K_m + K_d$), expressed as %].

With respect to the specificity of transport, Fig. 3 shows that GlySar (a dipeptide substrate of PepT1) and cefadroxil (an aminocephalosporin substrate of PepT1) could inhibit the permeability of valacyclovir by 50 and 80%, respectively, during jejunal coperfusions. In contrast, coperfusions of valacyclovir with L-valine (an amino acid substrate of ATB$^{0,+}$), L-histidine (an amino acid substrate of PhT1/2), PAH (an organic anion substrate of OATs), or TEA (an organic cation substrate of OCTs) were without effect. These results demonstrated that the jejunal permeability of valacyclovir was mediated by PepT1 and not by other putative transporters in the small intestine.

The jejunal permeability of acyclovir was also evaluated in wild-type and *PepT1* knockout mice. We found there was no difference in permeability between genotypes (4.0 ± 3.7 × 10^{-6} cm/s and 7.4 ± 7.3 × 10^{-6} cm/s, respectively, n = 4) and that acyclovir permeability was negligible compared with the jejunal permeability of valacyclovir in wild-type mice (1.7 ± 0.2 × 10^{-4} cm/s, n = 4–5).

In Situ Single-Pass Segment-Dependent Perfusion Studies. The quantitative contribution of PepT1 to valacyclovir regional permeability was probed in the small and large intestines of both genotypes. As demonstrated in Fig. 4, the permeability of 100 μM valacyclovir was segment dependent in wild-type mice, with mean P_{eff} values of 2.4 × 10^{-4} cm/s in duodenum, 1.7 × 10^{-4} cm/s in jejunum, 2.1 × 10^{-4} cm/s in ileum, and 0.27 × 10^{-4} cm/s in colon. Whereas there were no statistical differences in P_{eff} between the different segments of small intestine, these values were substantially higher than the P_{eff} observed in colon. Moreover, the P_{eff} of valacyclovir in duodenal, jejunal, and ileal segments of *PepT1* knockout mice were less than 10% of that in wild-type animals. Valacyclovir P_{eff} was similarly low in the colon of both genotypes. There were no statistical differences in
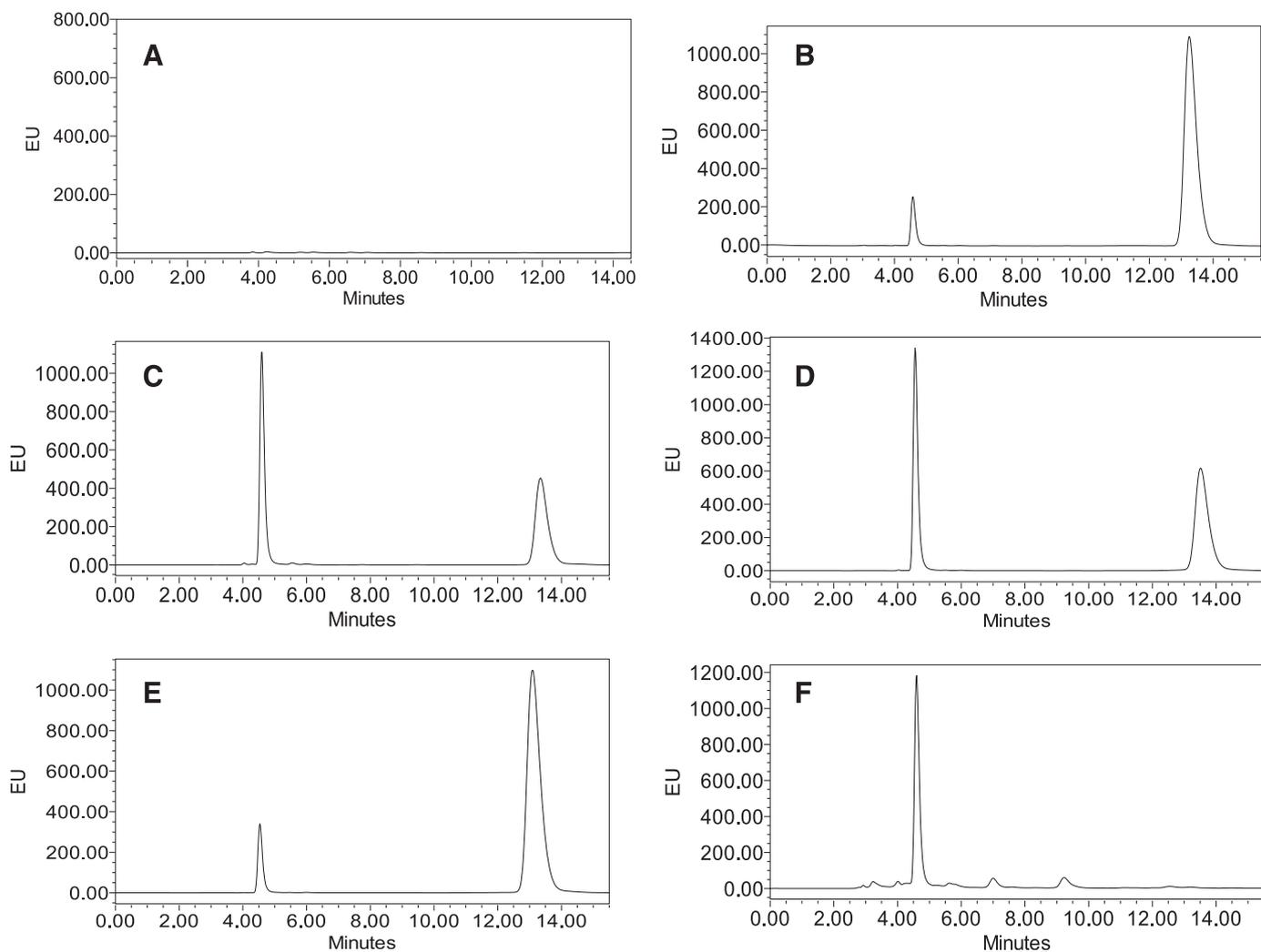
Fig. 1. Chromatograms of acyclovir and valacyclovir from blank perfusate (A), duodenal perfusate (B), jejunal perfusate (C), ileal perfusate (D), colonic perfusate (E), and portal vein plasma (F). The retention times are 4.6 minutes for acyclovir and 13.4 minutes for valacyclovir.

the P_{eff} of valacyclovir between any of the intestinal regions of *PepT1* knockout mice.

Oral Absorption Studies of Valacyclovir. Marked differences were observed between wild-type and *PepT1* knockout mice in this preliminary pharmacokinetic evaluation of acyclovir after oral administration of 25 nmol/g valacyclovir (Fig. 5). In particular, the C_{max} was 4.6-fold lower (2.0 $\pm$ 0.5 μM in *PepT1* knockout versus 9.3 $\pm$ 1.1 μM in wild-type animals, $n = 3$; $P \leq 0.01$), the T_{max} was 2.8-fold higher (70 $\pm$ 10 minutes in *PepT1* knockout versus 25 $\pm$ 5 minutes in wild-type animals, $n = 3$; $P \leq 0.05$), and the area under the acyclovir plasma concentration-time curve from time 0 to 180 minutes (a measure of systemic exposure) was 2.5-fold lower (301 $\pm$ 28 minutes·μM in *PepT1* knockout versus 741 $\pm$ 15 minutes·μM in wild-type animals, $n = 3$; $P \leq 0.001$) during PepT1 ablation. These results, and Fig. 5, demonstrate that mice deficient in PepT1 have a significantly reduced rate and extent of absorption of prodrug along with reduced peak and systemic concentrations of active drug after oral dosing of valacyclovir.

Discussion

The PepT1-mediated uptake of valacyclovir has been demonstrated in hPepT1-overexpressing Caco-2 cells (Han et al., 1998), hPepT1

cRNA-injected Xenopus oocytes (Balimane et al., 1998), hPepT1-overexpressing Chinese hamster ovary cells (Guo et al., 1999), and stably transfected hPepT1-transfected Madin-Darby canine kidney cells (Bhardwaj et al., 2005). However, the precise contribution of PepT1, compared with other transporters and uptake processes in mammalian intestine, is not possible by such in vitro studies. In this study, we quantitatively determined the contribution of PepT1 in the intestinal permeability of valacyclovir, via an in situ perfusion model, in wild-type and *PepT1* knockout mice. The results were further validated by in vivo experiments probing the oral absorption of valacyclovir in the presence and absence of PepT1. Our findings are novel and demonstrate for the first time that PepT1 accounted for 90% of the uptake for valacyclovir in small intestine; the permeability of valacyclovir was similar in duodenum, jejunum, and ileum, but much larger than colon which did not show any difference in prodrug uptake between genotypes; the jejunal permeability of valacyclovir was specific for PepT1 and saturable with a K_m of 10.2 mM; and the presence of intestinal PepT1 substantially improved the oral absorption of valacyclovir and systemic exposure of the active drug acyclovir.

The similarity of high permeability values for valacyclovir in duodenum, jejunum, and ileum are reflective of the similarity of high PepT1 protein levels found in these regions of the small intestine
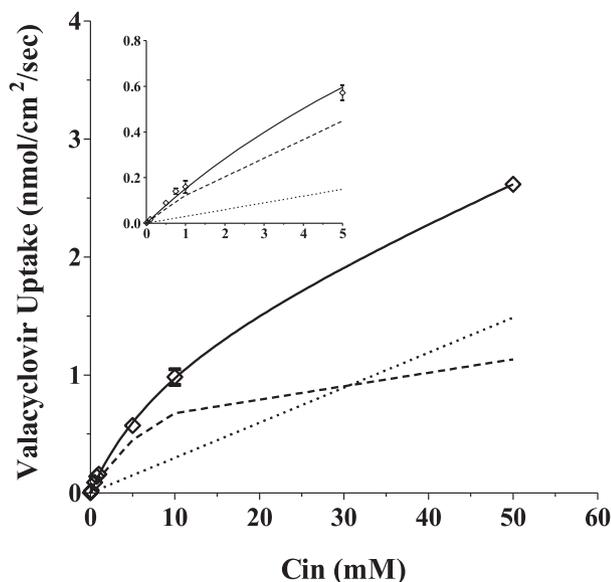
Fig. 2. Concentration-dependent uptake of valacyclovir (0.01–50 mM) during jejunal perfusions (pH 6.5) in wild-type mice. The lines represent simulations based on nonlinear Michaelis-Menten uptake (dashed), linear nonsaturable uptake (dotted), and total uptake (solid). The inset shows this relationship at lower concentrations (0.01–5 mM). Data are expressed as mean ± S.E. (n = 4–5). Cin, the inlet concentration of valacyclovir.

(Jappar et al., 2010). This is also true of the very low values for colonic permeability of valacyclovir and the very low, if any, expression levels of PepT1 protein in the colon. Thus, there appears to be a good correlation between the regional expression of PepT1 protein and the regional permeability of valacyclovir along the intestinal tract, as noted before for GlySar (Jappar et al., 2010). Domination of PepT1 in the small intestinal uptake of valacyclovir (i.e., 10% residual in null mice), along with its poor uptake in colon (i.e., only 11–16% of small intestine), suggests that the contribution of other possible intestinal transporters and/or uptake mechanisms is minimal at best. This finding is supported by the fact that L-valine, L-histidine, PAH, and TEA were without effect during jejunal
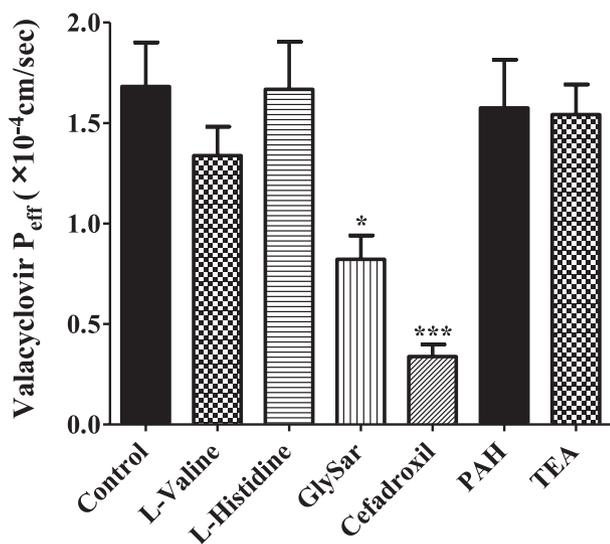
Fig. 3. Effect of potential inhibitors (25 mM) on the permeability of 100 μM valacyclovir during jejunal perfusions (pH 6.5) in wild-type mice. Data are expressed as mean ± S.E. (n = 4–5). *$P \leq 0.05$; ***$P \leq 0.001$ compared with control values.
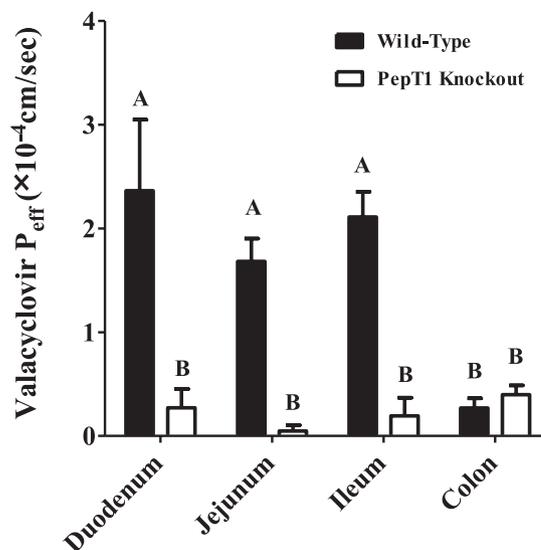

Fig. 4. Permeability of valacyclovir in different intestinal segments of wild-type and *PepT1* knockout mice. Data are expressed as mean ± S.E. (n = 4–5). Intestinal segments with the same capital letter (A or B) were not statistically different.

perfusions, thereby ruling out a significant contribution of $ATB^{0,+}$, PhT1/2, and those OATs and OCTs that have PAH and TEA as their respective substrates. It should be noted, however, that other investigators have observed a significant inhibitory effect of valacyclovir permeability by PAH or TEA during single-pass jejunal perfusions in rat (Sinko and Balimane, 1998). The reason for this contradictory result is unknown but may be related to differences in transporter expression between species (i.e., rat versus mouse) and differences in experimental design including buffer pH.

Valacyclovir was reported to be transported by the Na^+- and Cl^--coupled amino acid transporter $ATB^{0,+}$, as demonstrated in transfected human retinal pigment epithelial cells and cRNA-injected Xenopus oocytes (Hatanaka et al., 2004). Localized at the luminal membrane, $ATB^{0,+}$ was expressed in the distal but not proximal region of small intestine, and extensively in the colon of mice (Hatanaka et al., 2002). Based on these findings, the authors suggested that the potential of $ATB^{0,+}$ as a drug delivery system was at least comparable, if not greater, with that of PepT1 (Hatanaka et al., 2004). Our results do not support this contention since the colonic permeability of valacyclovir was so low compared with its uptake in the small intestine. Moreover, after colonic administration of 20 mg/kg valacyclovir to rats, there was no indication of prodrug absorption as evidenced by the lack of detectable plasma levels of acyclovir (Kagan and Hoffman, 2008). Finally, in light of the extensive luminal degradation of valacyclovir (Granero and Amidon, 2006) and the high fraction of acyclovir excreted in feces after oral dosing (de Miranda et al., 1981), it is unlikely that the colonic transporter $ATB^{0,+}$ would make a meaningful contribution to the intestinal absorption of valacyclovir.

As previously demonstrated, the Michaelis constant (K_m) of valacyclovir was quite variable among different experimental systems and species, with values of 0.3 mM (Han et al., 1998) and 1.6 mM (Bhardwaj et al., 2005) in Caco-2 cell cultures, 5.9 mM in hPepT1 cRNA-injected Xenopus oocytes (Balimane et al., 1998), and 3.8-5.0 mM in *hPepT1*-MDCK cells (Bhardwaj et al., 2005). The intrinsic K_m of valacyclovir was 1.2 mM in single-pass perfusions of rat jejunum (Sinko and Balimane, 1998). Moreover, the K_m of valacyclovir in Chinese hamster ovary cells transfected with the *hPepT1* gene was pH dependent and ranged from 7.4 mM (buffer pH 5.5) to 2.2 mM (buffer
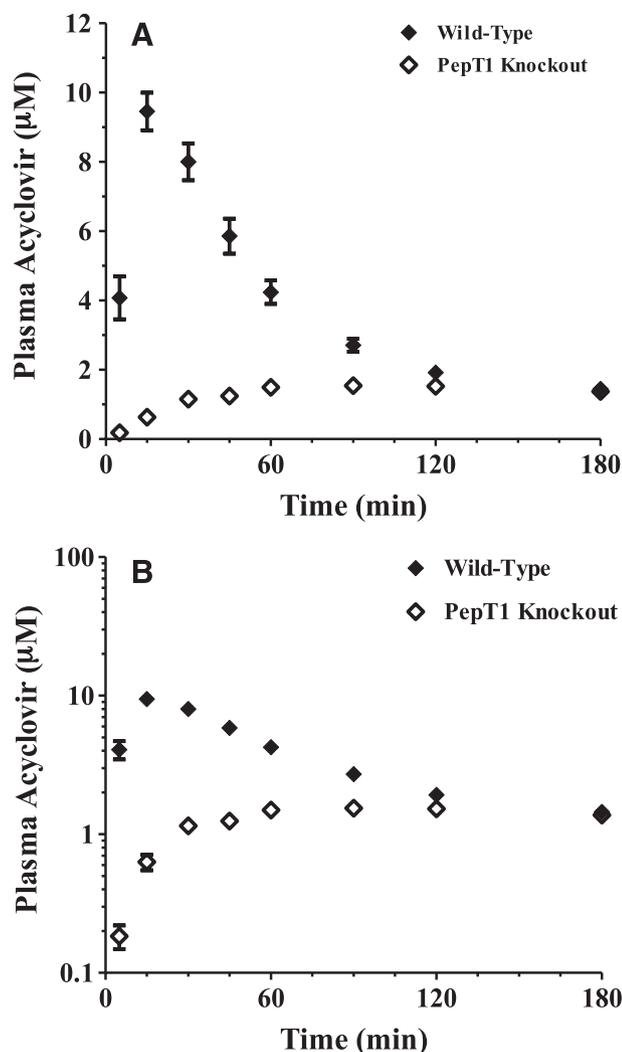
Fig. 5. Acyclovir plasma concentration-time curves in wild-type and *PepT1* knockout mice after oral administration of [³H]valacyclovir at a dose of 25 nmol/g. Data are expressed as mean ± S.E. (n = 4–7) in which the y-axis is displayed on a linear scale (A) and on a logarithmic scale (B).

pH 7.9), reflecting the increased percentage of neutral species of valacyclovir as the pH increased (Balimane and Sinko, 2000). Thus, our K_m of 10.2 mM for valacyclovir in jejunal uptake studies is consistent with the low-affinity transport characteristics of PepT1 and is similar to literature values, especially since it reflects concentrations in the bulk intestinal fluid rather than concentrations at the intestinal wall. In considering the contribution of active transport (V_{max}/K_m) versus that of the linear nonsaturable component (K_d), it is clear that PepT1 was the major factor responsible for the intestinal uptake of valacyclovir, accounting for 82% of all uptake processes.

The permeability of valacyclovir in our in situ jejunal perfusion studies in wild-type mice (1.68×10^{-4} cm/s) was essentially the same as the in vivo permeability of valacyclovir in humans (1.66×10^{-4} cm/s) (Lennernäs, 2007). Moreover, these values were similar to the in vivo permeability of metoprolol in human (1.34×10^{-4} cm/s) (Lennernäs, 2007) and in situ permeability of metoprolol in mouse (0.5×10^{-4} cm/s) (Jappar, 2009), a model compound for studying transcellular passive diffusion and one that is used as a reference for Biopharmaceutics Classification System class 1 drugs (i.e., high permeability and high solubility) (Benet et al., 2011). This comparison clearly demonstrates that the prodrug approach of targeting valacyclovir

to intestinal PepT1 effectively overcame the permeability barrier of the Biopharmaceutics Classification System class 4 drug acyclovir (i.e., low permeability and low solubility). This comparison also suggests that in situ single-pass intestinal perfusion studies in mice are a relevant experiment tool in estimating in vivo permeability values in humans.

Studies examining the in vivo pharmacokinetics of valacyclovir in wild-type and *PepT1* knockout mice corroborate our in situ perfusion studies and support the importance of intestinal PepT1 in enhancing the oral performance of this prodrug. This contention is demonstrated by the 4- to 5-fold greater peak plasma concentrations and 2- to 3-fold greater systemic exposures of acyclovir after valacyclovir oral dosing. The differences in exposure of acyclovir were a result of changes in absorption and not disposition since, after intravenous administration of [³H]acyclovir (25 nmol/g), the acyclovir plasma concentration-time profiles were virtually superimposable between wild-type and *PepT1* knockout mice (Yang et al., 2011). These results also illustrate that whereas our in situ studies may be valid mechanistically for valacyclovir, they do not necessarily reflect the degree of differences observed during more physiologic in vivo studies. Similar findings were reported for GlySar in which the oral absorption of dipeptide was only about 50% of that in *PepT1*-deficient versus *PepT1*-competent mice (Jappar et al., 2011; Ma et al., 2012) even though in vitro (Ma et al., 2011) and in situ (Jappar et al., 2010) experiments in jejunum showed a PepT1 contribution on the order of 80–90%. As suggested previously for GlySar, and again here for valacyclovir, we believe that these compounds take advantage of the intestine's residual length and long residence times so that passive absorption processes play a bigger role in the absence of PepT1. It should be appreciated that we could not differentiate between plasma acyclovir and valacyclovir in our study since radiolabeled prodrug was administered to the mice. However, as described previously, no valacyclovir was present in the portal vein during our in situ perfusion studies in jejunum. Based on this information and that, after oral administration, valacyclovir is essentially converted completely to acyclovir by presystemic intestinal and hepatic metabolism in rats, primates, and humans (Weller et al., 1993; Burnette and de Miranda, 1994; de Miranda and Burnette, 1994), it is very unlikely that valacyclovir contributes much, if anything, to the radioactive measurements in plasma.

In conclusion, our in situ and in vivo results in wild-type and *PepT1* knockout mice have provided definitive evidence for the significant contribution of PepT1 in improving the oral absorption of valacyclovir and the systemic exposure of acyclovir after oral valacyclovir administration. These findings strongly support the rationale for designing PepT1 targeted prodrugs for enhanced oral drug absorption in the gastrointestinal tract.

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